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Slug, a zinc finger gene previously implicated in the early patterning of the mesoderm and the neural crest, is also involved in chick limb development

Maria A. Ros[1], Mark Sefton[2] and M. Angela Nieto[2,*]

[1]Departamento de Anatomía y Biología Celular, Universidad de Cantabria, 39011 Santander, Spain
[2]Instituto Cajal, CSIC, Doctor Arce, 37, 28002 Madrid, Spain

[*]Author for correspondence (e-mail: anieto@cajal.csic.es)

SUMMARY

The great advances made over the last few years in the identification of signalling molecules that pattern the limb bud along the three axes make the limb an excellent model system with which to study developmental mechanisms in vertebrates. The understanding of the signalling networks and their mutual interactions during limb development requires the characterisation of the corresponding downstream genes. In this study we report the expression pattern of *Slug*, a zinc-finger-containing gene of the *snail* family, during the development of the limb, and its regulation by distinct axial signalling systems. *Slug* expression is highly dynamic, and at different stages of limb development can be correlated with the zone of polarizing activity, the progress zone and the interdigital areas. We show that the maintenance of its expression is dependent on signals from the apical ectodermal ridge and independent of Sonic Hedgehog. We also report that, in the interdigit, apoptotic cells lie outside of the domains of *Slug* expression. The correlation of *Slug* expression with areas of undifferentiated mesenchyme at stages of tissue differentiation is consistent with its role in early development, in maintaining the mesenchymal phenotype and repressing differentiation processes. We suggest that *Slug* is involved in the epithelial-mesenchymal interactions that lead to the maintenance of the progress zone.

Key words: *Slug*, limb patterning, progress zone, Sonic Hedgehog, chick embryo

INTRODUCTION

Our knowledge of the mechanisms underlying the patterning of the vertebrate embryo during development has advanced greatly with the molecular characterisation of certain key components. Recently, a further advance has been made with the demonstration that the same molecules may be used for the patterning of different regions or structures in the embryo. One example of this is Sonic hedgehog (SHH), a secreted molecule involved in the patterning of the central nervous system (Echelard et al., 1993; Roelink et al., 1995; Martí et al., 1995), the somites (Fan et al., 1994; Johnson et al., 1994) and the limb (Riddle et al., 1993; Chang et al., 1994; López-Martínez et al., 1995). Another example is *Slug*, a zinc finger-containing gene of the *snail* family, implicated in the epithelial-mesenchymal transitions that occur during early development, both in the formation of the mesoderm during gastrulation and in the emigration of neural crest cells from the neural tube (Nieto et al., 1994). Preliminary observations also showed specific domains of *Slug* expression during the early development of the limb. As discussed by Cohn and Tickle (1996), the limb is a good model system with which to study patterning mechanisms in vertebrates, since sets of molecules involved in the patterning of other embryonic structures are also present. In particular, the chick limb has the advantage of being easily accessible to experimental manipulations, allowing the expression of specific genes to be altered with subsequent analysis of the resulting phenotypes. Consequently, we chose to study the role of *Slug* during limb bud development of the chick embryo.

The development of the limb buds starts at stage 17 (Hamburger and Hamilton, 1951) with the formation of a bulge at the appropriate axial level of the lateral body wall. At stage 18, the ectoderm that covers the distal margin of the emerging bud differentiates into a special pseudostratified columnar epithelium called the apical ectodermal ridge (AER). The AER is a major signalling centre in the developing limb, being indispensable for the elongation of the bud (Saunders, 1948; Summerbell et al., 1973; Rowe and Fallon, 1982). Outgrowth and patterning in the proximo-distal axis (shoulder to fingers) depend on reciprocal interactions between the AER and the subjacent mesoderm (Saunders, 1948). Two other major signalling centres have been identified in the developing limb. One is the polarizing region (ZPA), a small mesodermal region localised at the posterior part of the bud. If a ZPA is transplanted to the anterior margin of another limb bud it produces mirror-image duplications (Saunders and Gasseling, 1968; Tickle et al., 1975), indicating its influence in the patterning of the antero-posterior axis (thumb to little finger). Finally, the control in the third, dorso-ventral axis (back to palm in the hand) appears to be regulated by the ectoderm (MacCabe et al., 1974; Geduspan et al., 1987).

During the last few years, advances have been made in the

molecular understanding of the signalling centres mentioned above (see Tickle, 1995). The action of the AER appears to be mediated by one or several members of the fibroblast growth factor (FGF) family. *Fgf-2*, *Fgf-4* and *Fgf-8* are all expressed in the AER and, furthermore, each of the three proteins FGF-2, FGF-4 and FGF-8, respectively, can substitute for the ridge (Niswander et al., 1993; Fallon et al., 1994; Mahmood et al., 1995). The mapped ZPA colocalises temporally and spatially with the area of expression of *Shh*, a vertebrate homologue of the *Drosophila* segment polarity gene *hedgehog*. Misexpression of *Shh* at the anterior margin or ectopic administration of SHH protein have the same effects as grafts of ZPA, indicating that SHH is the endogenous polarizing signal (Riddle et al., 1993; Chang et al., 1994; López-Martínez et al., 1995). Finally, WNT-7a and *Engrailed-1* are the candidate molecules for the action of the dorsal and ventral ectoderm on the dorso-ventral patterning respectively. WNT-7a is expressed in the dorsal ectoderm and has been shown to control dorsalisation by the induction of the LIM homeobox gene *Lmx-1* (Yang and Niswander, 1995; Parr and McMahon, 1995; Riddle et al., 1995; Vogel et al., 1995). *Engrailed-1* is expressed in ventral ectoderm and it has been shown to be essential for ventral limb patterning (Loomis et al., 1996).

Proper patterning and outgrowth of the limb require the coordinated action of the three signalling systems. A positive feed-back loop operates between the proximo-distal signalling system, mediated by one or several members of the FGF family, and the antero-posterior signal, mediated by SHH (Laufer et al., 1994; Niswander et al., 1994). In addition, the third axis mediating dorso-ventral patterning is mutually coordinated with the other two, as WNT-7a is also required to maintain *Shh* expression (Yang and Niswander, 1995). Thus, the adequate orchestration of the three signals or groups of signals appears to be required to properly activate downstream genes.

In the present work, we have analysed the expression of *Slug*, a member of the *snail* family of transcription factors (Nieto et al., 1994), during the development of the chick limb. We show that this gene exhibits a specific and dynamic pattern of expression during limb development. We have also analysed the regulation of *Slug* by the three signalling systems of the limb, trying to identify the position of *Slug* in the cascade of events that direct limb development.

MATERIALS AND METHODS

Preparation of embryos

Fertilised chicken embryos were purchased from Ibertek Farm, Valladolid, Spain and from Granja Rodriguez-Serrano, Salamanca, Spain. Eggs were routinely incubated, opened and staged according to Hamburger and Hamilton (1951). All the experimental manipulations were performed at stages ranging from 17 to 21. The specimens were subsequently fixed in 4% paraformaldehyde overnight and processed either for whole mount in situ hybridisation or embedded in Paraplast and serially sectioned (6 μm) for tissue section hybridisation.

Removal of the apical ectodermal ridge and FGF-2 bead implantation

The apical ectodermal ridge was removed from the right wing bud of stage 18-21 embryos with a sharpened tungsten needle. After the operation, the eggs were sealed with tape and returned to the incubator for 24 or 48 hours. In some cases, FGF-2 (1 mg/ml, a gift of Dr G. Gimenez) was applied on heparin acrylic beads (Sigma, H5263). The beads were grafted posteriorly into the subridge mesoderm immediately after ridge removal. We used PBS-soaked beads as controls.

ZPA grafts to the anterior wing border

The ZPA was obtained from wing buds at stages 19 and 20 and the ectoderm removed by mild trypsin digestion. Small pieces of ZPA were grafted to the anterior border of stage 20 and 21 wing buds, following the procedure of Tickle (1981). After the operation the eggs were sealed and returned to the incubator until the embryos were fixed.

Retinoic acid-soaked bead application

Retinoic acid (RA; all-trans-retinoic acid, from Sigma)-soaked beads (AG1X2, Bio-Rad) were implanted at the anterior border of wing buds, under the AER. Briefly, the beads were soaked in 0.1 mg/ml or 1 mg/ml RA in dimethyl sulfoxide (DMSO) for 20 minutes at room temperature, rinsed several times in PBS, and maintained for 20 minutes in PBS at 37°C before use. Control beads soaked in DMSO never gave rise to morphological alterations or modifications in gene expression.

Removal of the dorsal ectoderm

To remove the dorsal ectoderm of the wing bud, a cut was made in the dorsal ectoderm adjacent to the AER. Starting at the level of the cut, the ectoderm was peeled away towards the proximal part of the limb. After the operation, the eggs were sealed and returned to the incubator until the embryos were used.

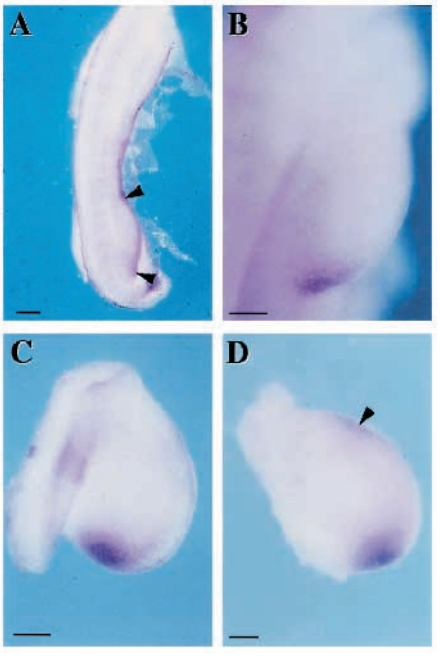

Fig. 1. Expression of *Slug* during early stages of limb development. (A) Dorsal view of a 34-somite embryo showing *Slug* expression in the ectoderm of the limbs and flank and also in the anterior and posterior mesoderm of the leg bud (arrowheads). (B) Dorsal view of a stage-19 wing bud showing *Slug* expression restricted to the posterior margin of the bud. (C) Ventral view of a stage-21 wing bud illustrating the increase in the area of *Slug* expression at the posterior border (compare with B). (D) Dorsal view of a slightly older wing bud showing the appearance of expression at the anterior border (arrowhead). All specimens are oriented with anterior towards the top. The bar indicates 250 μm.

For each type of manipulation, we operated on the right wing bud, leaving the unoperated left wing as a control. In each experiment, some embryos were allowed to develop for a further 7 days (a total of 11 days) to assess the effect of the manipulation. These embryos were dissected out, fixed in 10% formalin, stained with Victoria blue, cleared in methyl salicylate, and the pattern of the digits analysed.

In situ hybridisation in whole embryos and tissue sections

Digoxigenin-labelled antisense riboprobes were prepared and used for whole mount in situ hybridisation according to the procedure of Nieto et al. (1996). For the preparation of ^{35}S-labelled riboprobes and hybridisation in tissue sections, we followed the protocol described in Wilkinson and Nieto (1993). The *Slug* probe corresponded to nucleotides 1-358 of the cDNA (Nieto et al., 1994). Other probes used were *Shh* (kindly provided by T. Jessell), *Wnt-7a* (kindly provided by C. Tabin), *Msx1* (kindly provided by B. Robert) and *Msx2* (kindly provided by A. Kuroiwa).

Double in situ detection of *Slug* transcripts and DNA fragmentation

We analysed the distribution of apoptotic cells in paraffin sections of embryos previously hybridised with the *Slug* probe in whole mounts. In situ hybridisation was carried out as described above, and the stained embryos were subsequently fixed in 4% paraformaldehyde, embedded in paraffin and sectioned at 15 μm (Nieto et al., 1996). To detect genomic DNA breaks we used terminal transferase to incorporate fluorescein-dUTP, which was visualised with a POD-conjugated antifluorescein antibody ('In situ cell death detection kit, POD', Boehringer-Mannheim), following the manufacturers instructions.

RESULTS

Expression of *Slug* in the developing wing and leg

Slug expression was first detected in the ectoderm covering the limb bud and flank at stage 18 (Fig. 1A). At this stage some expression was also detected in the mesenchyme at the anterior and posterior border of the leg bud (arrowheads in Fig. 1A). At stages 19 and 20, the expression of *Slug* appeared to be confined to the posterior area of the wing bud, overlapping the ZPA (Fig. 1B). Transverse sections of the limb at these stages showed that the expression of *Slug* was stronger in the mesenchyme but that it could also be detected in the ectoderm (not shown). At stage 21, the mesodermal domain of *Slug* expression extended distally under the AER into the progress zone (Fig. 1C) and, subsequently, *Slug* expression was also detected at the anterior border of the bud (Fig. 1D). At stage 23-24 the whole periphery of the bud expressed *Slug* at high levels (Fig. 2A,B). As at previous stages, *Slug* expression was stronger in the mesoderm but was also detected in the dorsal ectoderm (Fig. 2C). However, the AER did not show detectable *Slug* expression at any stage examined. The pattern of *Slug* expression spanning the periphery of the limb bud can be seen

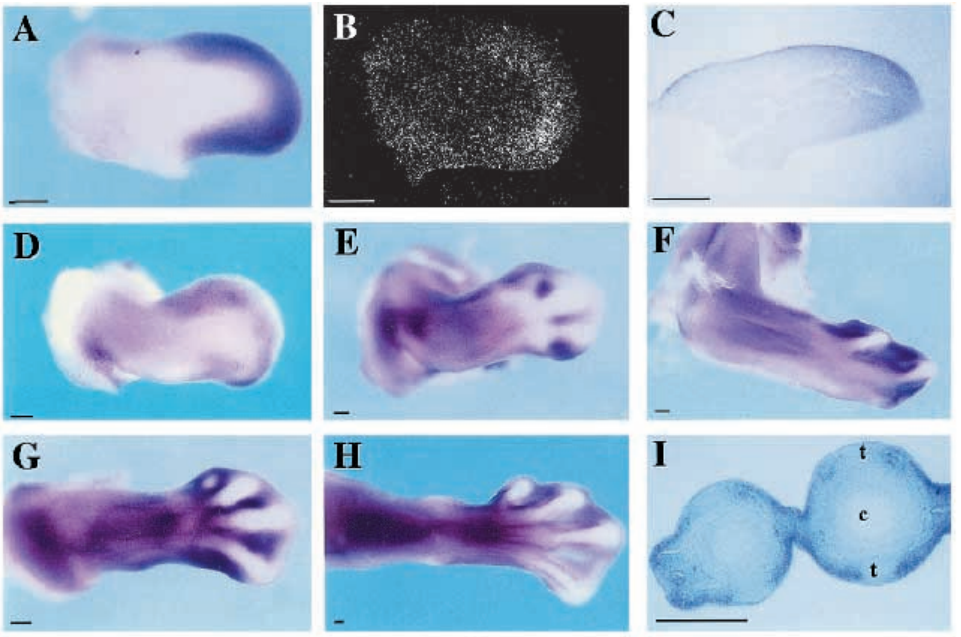

Fig. 2. Expression of *Slug* at later stages of wing and leg bud development. (A) *Slug* is expressed in the periphery of stage-24 wing buds. (B) The radioactive hybridisation of a frontal section of a stage-24 wing bud demonstrates the mesenchymal nature of *Slug* expression. (C) Longitudinal section through the posterior part of the bud shown in A, where the expression of *Slug* by the dorsal ectoderm can be clearly appreciated. (D) Expression of *Slug* in a stage-26 wing bud showing the distribution of transcripts at the periphery of the limb. (E) Expression of *Slug* in a stage-28 wing bud. Strong expression is observed in the interdigital areas and in the anterior and posterior margins of the limb. (F) Stage-32 wing hybridised with *Slug*, showing continued expression in the interdigital areas. (G,H) Expression of *Slug* in the interdigital areas of stage 29 (G) and 32 (H) leg buds. (I) Transverse section of a stage-32 leg bud at the level of the interdigits. Expression of *Slug* is undetectable in the cartilage (c) or tendon (t) condensations. All limb buds are oriented with anterior to the top except the sections, which are oriented with dorsal to the top. Bar, 250 μm.

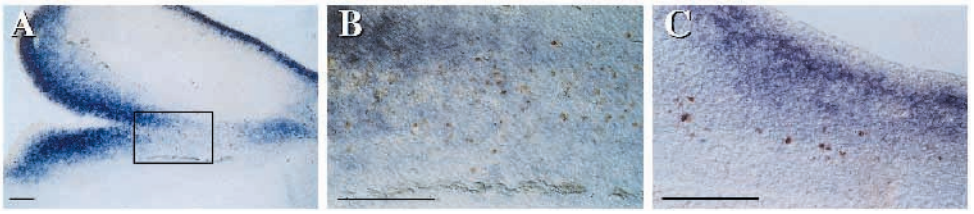

Fig. 3. *Slug* expression and interdigital cell death. (A) Double labelling for *Slug* expression and in situ detection of DNA breaks in a frontal section through the first interdigit of a stage-32 leg bud. *Slug*-expressing cells, seen in blue-purple, and apoptotic cells, labelled in brown, do not colocalise. (B,C) High-power photographs of the interdigital area (insert in A) and anterior margin, respectively, to show that most of the dying cells are separated from the areas of *Slug* expression. Bar, 500 μm.

until stage 26 (Fig. 2D). From stage 28, when the developing digits and toes became clearly distinct, elevated *Slug* expression was observed in the interdigital areas while the digital rays lacked detectable *Slug* expression (Fig. 2E,F). The expression of *Slug* in the leg followed the same pattern described for the wing but slightly delayed (Fig. 2G-I). Interestingly, *Slug* expression during limb development was always associated with areas of undifferentiated mesoderm and was undetectable in the cartilaginous condensations (Fig. 2H,I) or other regions of cell differentiation such as tendon condensations (Fig. 2I).

Slug expression and interdigital cell death

During chick limb bud development, four well-defined areas of programmed cell death have been described (Saunders and Fallon, 1967; see Hinchliffe, 1982 and Hurlé et al., 1995). Interdigital cell death is the last to appear during limb development and constitutes the so-called interdigital necrotic zones (INZ). The INZ are areas of massive mesenchymal cell death located between the digits, essential for the individualisation or separation of the digits (Saunders and Fallon, 1967; Pautou, 1975). Despite their name (given before the introduction of the term apoptosis) the cells in these areas die by apoptosis (Garcia-Martínez et al., 1993; Mori et al., 1995; Hurlé et al., 1995). Not all the cells in the interdigit die at the same time, but there is a specific pattern of cell death that has been well documented (Pautou, 1975). As we have described above, the expression of *Slug* in the interdigital areas is very high (Fig. 2E-H). Consequently, we decided to analyse the possible relationship between cell death and *Slug* expression. We performed in situ detection of DNA breaks by the TUNEL method in sections of limbs previously hybridised for *Slug* in whole mounts (see Materials and Methods). This approach allowed us to identify *Slug*-expressing cells and dying cells in the same section. At stage 29, before the initiation of the interdigital cell death, *Slug* transcripts are found in the whole interdigital area (Fig. 2G). However, by stage 32, when interdigital apoptosis is intense, the expression of *Slug* appears to concentrate in the periphery of the interdigital area, leaving the central region almost devoid of transcripts (Fig. 2H). Concomitant with interdigital cell death, apoptosis also occurs at the anterior and posterior margins of the autopod. Fig. 3A-C shows that most of the apoptotic cells, both at the INZ (Fig. 3B) and at the margin (Fig. 3C) do not coincide with areas of *Slug* expression. Thus, we observed no direct relationship between the expression of *Slug* and apoptosis, indeed it appeared that when the cells start to die they do not express *Slug*.

Regulation of *Slug* expression by the apical ectodermal ridge

We have analysed the regulation of *Slug* expression by the three signalling systems of the developing limb. Removal of the AER leads to the development of truncated limbs, whereby the earlier the ridge is removed, the greater the truncation obtained (Fig. 4A,B). The removal of the AER at early stages of limb development (from stages 18 to 20) caused down-regulation of the mesenchymal expression of *Slug*, which became undetectable 24 hours after the operation (Fig. 4C,D). However, the dorsal ectodermal expression of *Slug* was maintained and appeared as a distal blue rim around the truncated limb in the whole mounts (arrowheads in Fig. 4C). Although at lower levels, transcripts can also be detected in the ventral ectoderm, suggesting that the removal of the apical ridge may allow the spreading of *Slug* expression to more ventral regions (Fig. 4D-F). In some cases, we hybridised consecutive serial sections for *Slug*, *Msx-1* and *Msx-2*. The apical ridge cells express high levels of *Msx-2* (Fig. 4G), allowing us to determine whether the ridge had been properly removed. *Msx-1* expression in the subridge mesoderm is highly dependent on the presence of the ridge, becoming undetectable after its removal (Fig. 4H).

Once we had demonstrated that *Slug* expression was dependent on the apical ridge, we asked whether FGF-2 could maintain *Slug* expression, as FGFs have been shown to be able to substitute for the action of the AER. To check this, we implanted FGF-2-soaked beads (1 mg/ml) at different positions of the progress zone, immediately after the removal of the AER. Our results showed that FGF-2 maintained *Slug* expression in the mesoderm adjacent to the bead. When the FGF-2 bead was implanted posteriorly, *Slug* expression was maintained posteriorly (Fig. 5A). If the FGF-2 bead was implanted anteriorly, *Slug* expression was maintained anteriorly (Fig. 5B) and if the bead was implanted mid-distally, *Slug* expression was maintained distally around the bead (not shown). To analyse whether FGF-2 was not only able to maintain *Slug* expression but also to induce it, we implanted FGF-2 beads at positions where there is no endogenous expression. FGF-2 beads implanted into the mid-proximal mesoderm did not induce detectable *Slug* expression after ridge removal (Fig. 5C) or in unoperated limbs (Fig. 5D).

Regulation of *Slug* expression by the zone of polarizing activity

At early stages of limb development (stages 19-21) the expression of *Slug* colocalises with the ZPA. The action of the ZPA appears to be mediated by SHH, whose expression is first detected at stage 17 (Riddle et al., 1993). Therefore, *Shh* expression precedes *Slug* expression, suggesting that *Slug* expression could be regulated by SHH. To analyse this possibility, we grafted a piece of the polarizing region to the anterior border of the developing wing bud. In similar experiments, the grafted ZPA induces expression of the most 5′ *Hoxd* genes within 24-48 hours (Izpisúa-Belmonte et al., 1991; Nohno et al., 1991). In our experiments, the grafted ZPA did not induce *Slug* expression in the anterior mesoderm after 24 hours. The expression pattern obtained in the experimental wing was similar to that of the control wing except for the presence of transcripts in the graft itself (Fig. 6A). In every case analysed 48 hours after the operation, the expression of *Slug* was continuous across the periphery of the limb with the experimental limb showing evidence of limb duplication (not shown).

It has been shown that retinoic acid (RA), when applied to the anterior border of the wing bud, is able to mimic the action of the ZPA through the induction of a new polarizing region (Wanek et al., 1991). When RA was applied anteriorly to the limb, the pattern of *Slug* expression after 24 hours was different from the one obtained following ZPA grafts. The two different concentrations of RA used (1 mg/ml and 0.1 mg/ml) gave identical results. RA beads prevented the dynamic progression of *Slug* expression so that it remained confined to the postero-distal part of the bud (Fig. 6B). The inhibition of the expression of *Slug* by RA at the anterior border was, however, overridden after 48 hours, when its domain of expression

spanned the distal periphery of the limb. At this time the shape of the limb bud already exhibited signs of the duplication (not shown).

Regulation of *Slug* expression by the dorsal ectoderm

The dorsal ectoderm of the limb appears to control patterning in the dorso-ventral axis (Yang and Niswander, 1995; Parr and McMahon, 1995). Signalling in this axis is linked to the signalling in the anterior-posterior axis, as the dorsal ectoderm is required to maintain *Shh* expression in the limb bud. When the dorsal ectoderm is removed, *Shh* expression greatly decreases and the bud that develops lacks the ulna and the fourth digit (Fig. 7A; Yang and Niswander, 1995). Thus, we analysed the dependence of *Slug* expression on the dorsal ectoderm. We removed the dorsal ectoderm and analysed *Slug* expression 24 hours after the operation. Whole mount in situ hybridisation showed that the removal of the dorsal ectoderm did not affect the pattern of *Slug* expression but that the level of expression increased (Fig. 7B,C). This effect was detected in embryos subjected to the removal of the dorsal ectoderm from stage 17 to stage 21. In distal transverse sections of the hybridised wing buds, we were able to more clearly observe the increase in *Slug* expression, as seen in Fig. 7D and E, both in the dorsal mesoderm under the removed ectoderm, and in the ventral mesoderm. We also hybridised consecutive serial sections with *Wnt-7a*, *Slug* and *Shh*. These sections showed that *Slug* expression was maintained**,** although it was difficult to evaluate whether or not there was an increase in the level of expression (Fig. 7F). Hybridisation with *Wnt-7a* demonstrated the extent of dorsal ectoderm removal, also manifested by the great reduction of *Shh* expression (Fig. 7G,H). Therefore, the maintenance of *Slug* expression does not require signalling from the dorsal ectoderm, nor does it require normal levels of SHH.

DISCUSSION

The recruitment of the same molecules for the patterning of different structures at different stages of embryonic development is a strategy widely used throughout animal phyla. *Slug* is a zinc finger-containing gene previously shown to be involved in the delamination of the early mesodermal cells from the primitive streak at gastrulation and of the neural crest cells from the neural tube (Nieto et al., 1994). It is a member of the *snail* family of transcription factors, *snail* being required for mesoderm formation in the gastrulating *Drosophila* embryo, by inhibiting the expression of non-mesodermal genes (Leptin, 1991). Later on in fly development, *snail* has a second phase of expression in the nervous system (Alberga et al., 1991). The mouse homologue of the *snail* gene, *Sna*, is also expressed in the mesoderm at gastrulation stages, suggesting the putative conservation of its early role from insects to mammals. However, later on it is expressed in precartilage, suggesting an involvement in chondrogenesis throughout the embryo, including the formation of cartilage in the limb (Nieto et al., 1992). In this work, we report that another family member, *Slug*, apart from its role in the early patterning of the mesoderm and the neural crest, is also involved in the patterning of the chick limb.

Slug pattern of expression during chick limb development

During the development of the limb, *Slug* is expressed in both the mesoderm and the ectoderm. The mesodermal domain of *Slug* expression initially colocalises with the ZPA. During successive stages, this domain progressively expands into the progress zone and the anterior border. *Slug* expression is dynamic, varying from stage to stage, but it is always excluded from areas undergoing cell differentiation. Depending on the stage, the expression of *Slug* may be correlated with the ZPA, the progress zone and, at later stages, with the interdigital areas.

Slug expression was detected in the dorsal ectoderm during the whole period analysed. It is difficult to ascertain the relevance of the ectodermal expression of *Slug*. Nevertheless, it should be noted that *Slug* expression in the ectoderm is not affected by any of the experimental manipulations performed in this study, indicating that ectodermal and mesodermal domains of *Slug* expression are regulated independently.

In order to gain insights into the functional implication of *Slug* expression, we have analysed its association with the three major signalling systems of the developing limb.

Regulation of *Slug* expression by the ZPA, the AER and the dorsal ectoderm

From stages 19 to 21, *Slug* expression is confined to the posterior mesoderm of the limb, colocalising with the ZPA. SHH is considered to mediate the action of the ZPA (López-Martínez et al., 1995) and is first detected at stage 17 (Riddle et al., 1993). *Slug* expression in the ZPA is first detected two stages later than *Shh*, suggesting that it may be downstream of SHH. However, this possibility seems to be unlikely because ZPA ectopically grafted to the anterior margin of the wing bud does not induce ectopic *Slug* expression, whereas it does induce *Bmp-2* and the most 5′ *Hoxd* genes, which are thought to be part of the SHH signalling pathway (Francis et al., 1994; Laufer et al., 1994). This result suggests that SHH may not be required for the initiation of *Slug* expression. Furthermore, SHH (at least at its normal levels of expression, see below) does not appear to be required for the maintenance of *Slug* expression. Like the ZPA, application of RA at the anterior border does not induce *Slug* expression but, interestingly, inhibits the normal *Slug* expression at the anterior margin of the limb, indicating that RA can specifically inhibit *Slug* expression, at least at the concentrations used in this study. This result still supports the idea that RA is the endogenous signal for the induction of the ZPA if, as suggested by Helms et al. (1996), RA acts prior to stage 17, and the blocking of its signalling pathway has little effect in limb patterning at later stages. Helms et al. (1996) also show data in which the experimental rate of RA synthesis in the wing region is 12 times less at stage 20 than at stage 14. It is possible that this decay in RA levels allows cells to express *Slug*. In any case, it appears that the conditions required for the activation of *Shh* are not compatible with *Slug* expression.

From stage 21, *Slug* expression is progressively detected in the progress zone. The progress zone is an area of undifferentiated, rapidly proliferating mesenchymal cells located under the ridge, whose maintenance depends on the presence of the ridge (Summerbell et al., 1973). When the AER is removed, *Slug*

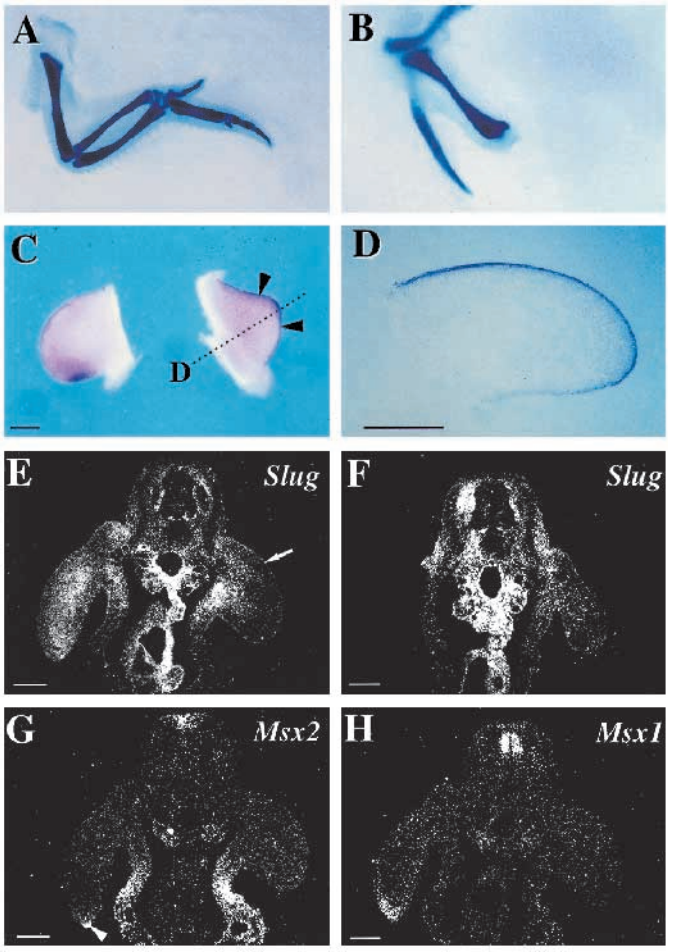

Fig. 4. Down-regulation of *Slug* expression after apical ridge removal. (A) Whole-mount cartilage staining of a normal day-11 embryonic wing. (B) Truncated limb at the level of the elbow obtained after the removal of the apical ridge at stage 20. (C) 24 hours after ridge removal at stage 20, *Slug* expression is undetectable in the mesoderm of the operated wing while ectodermal expression persists, giving rise to a blue rim of staining (arrowheads). This expression can be better assessed in the longitudinal section shown in **(**D). The dashed line in C indicates the approximate position of this longitudinal section. (E-H) Darkfield micrograph of adjacent transverse sections through an embryo (longitudinal section of the limbs) hybridised with *Slug* (E,F), *Msx-2* (G) and *Msx-1* (H), 24 hours after the apical ridge was removed at stage 20. (E) Note the lack of *Slug* expression in the mesoderm of the operated limb and the expression in the ectoderm (arrow). (F) *Slug* expression in the mesenchyme of the operated limb is also undetectable at the most posterior levels of the bud. (G) Note the high expression of *Msx-2* in the apical ridge (arrowhead) and the lack of the apical ridge in the operated limb. (H) *Msx-1* is undetectable in the subridge mesoderm of the experimental wing. In all cases the operated limb is to the right. Bar, 250 μm.

expression in the mesoderm is down-regulated so that it becomes undetectable 24 hours later. It is possible that some *Slug*-expressing cells in the progress zone die after ridge removal (Rowe et al., 1982). It is known that signals from the ridge and the ZPA act in concert, and that the removal of the AER rapidly down-regulates *Shh* expression (Laufer et al., 1994; Shaoguang et al., 1996). Consequently, the decay in *Slug* expression after AER removal could be mediated by *Shh* down-regulation. However, the dramatic decrease in *Shh* expression after dorsal ectodermal removal (Yang and Niswander, 1995) does not cause *Slug* down-regulation. These results indicate that the down-regulation in *Slug* expression seen after the removal of the AER is likely to be caused by the deprivation of ridge factors and that SHH may not be involved in this process. In this context, beads soaked in FGF-2, shown to be able to substitute for the ridge (Fallon et al., 1994), maintain *Slug* expression after AER removal. Our experiments demonstrate that FGF-2 is able to maintain *Slug* expression both at the anterior and posterior borders of the limb bud, in contrast to the maintenance of *Shh* expression only when this factor is present at the posterior border. We conclude that the maintenance of *Slug* expression is dependent on signals from the AER and independent of SHH. However, FGF-2 is unable to induce *Slug* expression in non-expressing mid-proximal mesenchyme.

The increase in the mesenchymal expression of *Slug* after dorsal ectoderm removal, also indicates that the dorsal ectoderm may down-regulate the mesodermal expression of *Slug*. The dorsal ectoderm expresses WNT-7a, a secreted protein involved in the regulation of the dorsoventral patterning of the limb

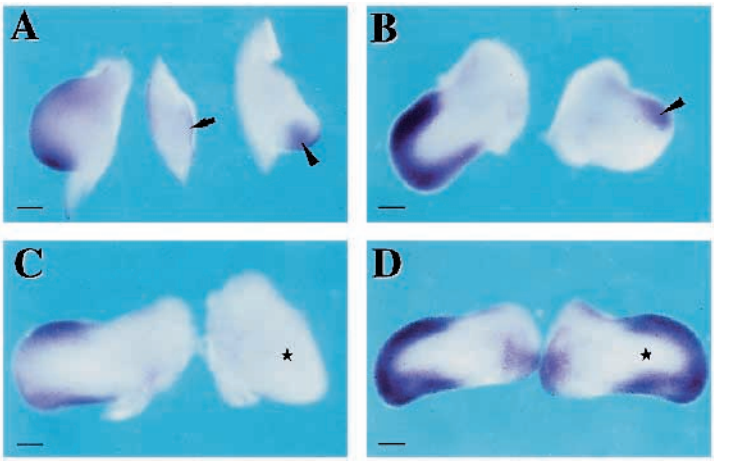

Fig. 5. FGF-2 maintains *Slug* expression after AER removal but does not induce it. (A) Posterior application of FGF-2 after ridge removal maintains the expression of *Slug* around the bead (arrowhead). This picture shows a control, unoperated wing (left), a wing 24 hours after removal of the ridge at stage 20 (middle, arrow indicates distal) and a third wing that received an FGF-2 bead immediately after the removal of the ridge at stage 20 (right). (B) *Slug* expression was also maintained when FGF-2 was applied into the anterior progress zone after the removal of the ridge at stage 21 (arrowhead). FGF-2 did not induce *Slug* expression in mid-proximal mesoderm after ridge removal (C) or in normal wings (D). The asterisks indicate the position of the bead. In all cases the embryos were analysed 24 hours after the operations and in the photographs, the operated limb is on the right. Bar, 250 μm.

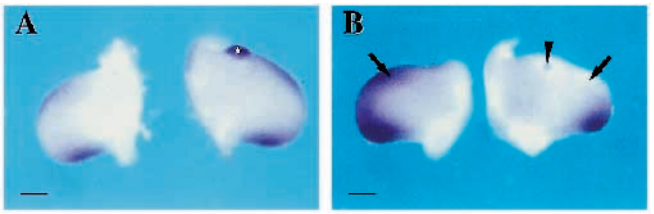

Fig. 6. Effects of anterior border grafts of ZPA and retinoic acid on *Slug* expression. (A) Graft of ZPA under the anterior apical ridge of a stage-20 wing bud showing *Slug* expression 24 hours after the operation. The asterisk indicates the position of the graft. (B) 24 hours after a retinoic acid bead (1 mg/ml, arrowhead) was placed at the anterior border of a stage-20 wing bud, there was no anterior induction of *Slug* expression and the normal expression at the anterior border (arrows) was inhibited. The operated limb is to the right. Bar, 500 μm.

through the induction of *Lmx1* in the dorsal mesoderm (Riddle et al., 1995; Vogel et al., 1995). Whether WNT-7a is the molecule responsible for the partial inhibition of *Slug* mesodermal expression by the ectoderm remains to be determined. The influence of the dorsal ectoderm on *Slug* expression is deeper, affecting both the dorsal and distoventral mesoderm, than the influence of WNT-7a in inducing *Lmx1*, which only affects the dorsal mesoderm (Riddle et al., 1995; Vogel et al., 1995).

Relationship of *Slug* expression with programmed cell death

At later stages of limb development, *Slug* is expressed in the interdigits and in the anterior and posterior margins of the autopod. The interdigits are areas where extensive apoptosis occurs and, consequently, genes expressed in these areas have been suggested to participate in the cell death programme. Among these genes are *Msx-1*, *Msx-2*, *Bmp-2* and *Bmp-4* (Hill et al., 1989; Francis et al., 1994; Zou and Niswander, 1996). *Slug* expression starts in the interdigital regions at about stage 28, well before the initiation of the cell death process. At this stage, *Slug* transcripts are found in the whole interdigit. Later on, when apoptosis is underway, *Slug* transcripts are detected mainly in the periphery of the interdigit, and not in association with the concomitant cell death. This observation suggests that cells down-regulate *Slug* expression before entering the cell death process. Several observations indicate that the interdigital cells are in an undifferentiated state (Gañán et al., 1996). *Slug* could be involved in specifying the undifferentiated state of the interdigit (see below). If this is the case, and taking into account that cell death can be considered as a 'differentiation' programme, it would be reasonable that cells down-regulate *Slug* expression when they are committed to die.

Putative roles of Slug in the patterning of the chick limb bud.

The correlation of *Slug* expression with areas of undifferentiated mesenchyme at stages in which the limb is undergoing tissue differentiation programmes, is in agreement with the expression of *Slug* during early stages of chick development. *Slug* is expressed in the early mesodermal cells delaminating from the primitive streak, and these cells maintain its expression while retaining the undifferentiated mesenchymal character (Nieto et al., 1994). In the same way, *Slug* is also expressed in the neural crest cells just before their emigration from the neural tube, and they continue to express it while migrating to their corresponding destinations (Nieto et al., 1994). This is consistent with a function in the maintenance of the mesenchymal phenotype and in the inhibition of differentiation. In this respect, *Slug* has been implicated in the epithelial-mesenchymal (EM) transition that occurs during the two early developmental processes mentioned above (Nieto et al., 1994; Sefton and Nieto, unpublished observations) indicate that it is also expressed in other regions of the embryo where EM transitions or EM interactions are taking place.

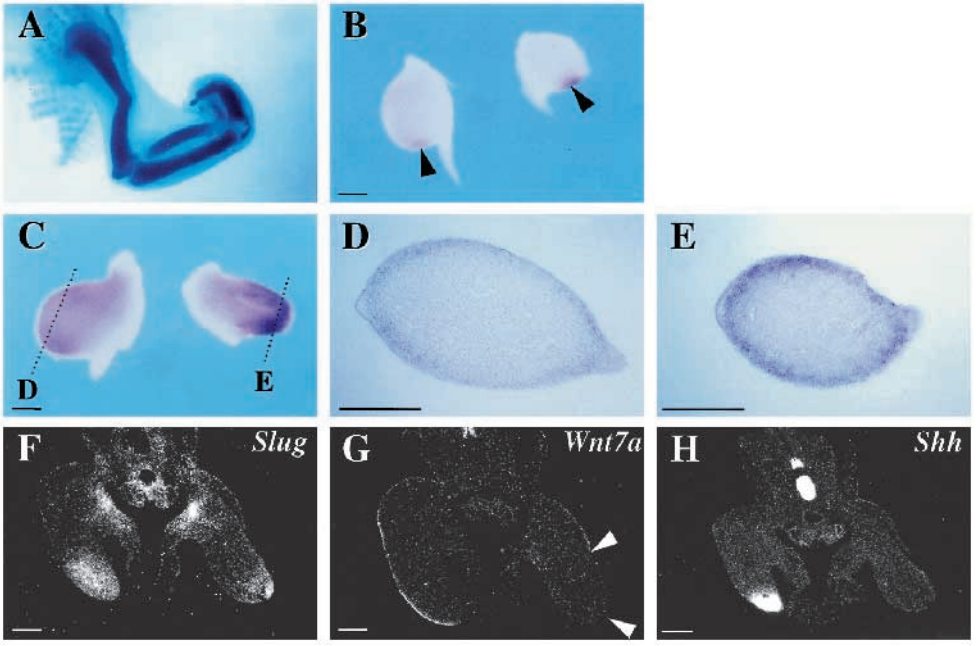

Fig. 7. Effect of dorsal ectoderm removal on the expression of *Slug*. (A) Example of a day-11 embryonic wing obtained after the removal of the dorsal ectoderm at stage 20, showing abnormal bending and lack of digit 4. (B) 24 hours after the removal of the dorsal ectoderm at stage 17, there is an increase in the level of *Slug* expression compared with the contralateral limb (arrowheads). Note that the pattern of expression is not modified. (C) Increase in the level of mesodermal expression of *Slug* 24 hours after the dorsal ectoderm was removed at stage 19. The dashed lines indicate the approximate positions of the transverse sections shown in D (control) and E (operated wing). (F-H) Darkfield micrographs of adjacent sections hybridised with *Slug* (F), *Wnt-7a* (G) and *Shh* (H), 24 hours after the removal of the dorsal ectoderm. The hybridisation with *Wnt-7a* (G) allows evaluation of the ectoderm removal, which is also confirmed by the reduction in *Shh* expression (H). The arrowheads in G demarcate the region deprived of dorsal ectoderm. In all the photographs the operated limb is to the right. Bar, 250 μm.

The main epithelial-mesenchymal interaction in the developing limb is that established between the AER and the progress zone, whereby a permissive signal from the ridge maintains the subapical mesoderm in an undifferentiated stage. This signal appears to be provided by one or several members of the FGF family. The feedback loop between the ectoderm and the mesoderm during limb patterning is presently being characterised at the molecular level. MSX-1 appears to be involved in the specification of the progress zone, possibly acting to repress transcription and maintain the cells in an undifferentiated state under the influence of the AER (Ros et al., 1992; Song et al., 1992; Catron et al., 1995). In this context, FGFs are able to maintain *Msx-1* expression in the progress zone after ridge removal (Fallon et al., 1994) and to reactivate its expression in proximal tissues (Kostakopoulou et al., 1996). Interestingly, FGF-2 is also able to maintain *Slug* expression after removal of the ridge. These observations and the fact that another family member, *snail*, is a transcriptional repressor (Leptin, 1991), lead us to speculate that, as MSX-1, SLUG could possibly act by repressing the expression of genes required for differentiation. We therefore suggest that SLUG could be involved in the epithelial-mesenchymal interactions that lead to the maintenance of the progress zone. However, it is unlikely that SLUG participates in its specification, since it is not detected in the progress zone until stage 21. It also seems likely that SLUG is involved in the interactions between the dorsal and ventral ectoderm and the subjacent mesoderm.

The coordinated expression of *Shh* and *Fgf-4* constitutes the feedback loop between the ZPA and the AER (Laufer et al., 1994; Niswander et al., 1994) that maintains proliferation and patterning throughout the progress zone. Very recently, BMP-2 has been shown to induce the expression of *Fgf-4* and *Hoxd* genes when ectopically expressed in the anterior mesenchyme of the wing bud (Duprez et al., 1996), indicating that SHH can activate the expression of these genes via BMP-2. However, in the *limbless* mutant, *Hoxd-11* and *Hoxd-12* genes are assymetrically expressed in the limb bud while *Shh* and *Bmp-2* expression remains undetectable (Ros et al., 1996). Nevertheless, assuming that SLUG might be involved in the maintenance of the progress zone, and trying to situate it in this signalling cascade, it is worth noting that BMPs are able to induce MSX and SLUG expression in ventral neural plate explants (Liem et al., 1995). Furthermore, at some stages of limb development, the pattern of expression of *Slug* parallels that of some of the BMPs (Francis et al., 1994). It is then tempting to suggest that *Slug* may be downstream of BMPs in the process of limb patterning, although further work will be necessary to demonstrate this.

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